Autophagy in Atherosclerosis: A Cell Survival and Death Phenomenon With Therapeutic Potential

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Abstract—Autophagy is a reparative, life-sustaining process by which cytoplasmic components are sequestered in double-membrane vesicles and degraded on fusion with lysosomal compartments. A growing body of evidence suggests that autophagy is stimulated in advanced atherosclerotic plaques by oxidized lipids, inflammation, and metabolic stress conditions. However, despite the increasing interest in autophagy in various pathophysiological situations such as neurodegeneration, cancer, and cardiac myopathies, the process remains an underestimated and overlooked phenomenon in atherosclerosis. As a consequence, its role in plaque formation and stability is poorly understood. Most likely, autophagy safeguards plaque cells against cellular distress, in particular oxidative injury, by degrading damaged intracellular material. In this way, autophagy is antiapoptotic and contributes to cellular recovery in an adverse environment. An interesting observation is that basal autophagy can be intensified by specific drugs. Excessively stimulated autophagic activity is capable of destroying major proportions of the cytosol, leading finally to type II programmed cell death that lacks several hallmarks of apoptosis or necrosis. Because atherosclerosis is an inflammatory disorder of the arterial intima, pharmacological approaches could be developed to stabilize vulnerable, rupture-prone lesions through selective induction of macrophage autophagic death. (Circ Res. 2009;104:304-317.)

Key Words: atherosclerosis ▪ autophagy ▪ autophagosome ▪ lysosome ▪ cell death

Atherosclerosis is a long-term inflammatory disease of the arterial wall and the leading cause of death and morbidity among adults in the developed countries.1-3 It slowly progresses over a period of decades before clinical symptoms become manifest. Progression of the disease is characterized by the formation of atherosclerotic plaques and depends on genetic make-up, sex, and certain well-recognized risk factors such as hypercholesterolemia, diabetes mellitus, hypertension, obesity, and smoking.4,5 Initially, plaque formation was viewed as an inevitable degenerative process. Nowadays, our knowledge about plaque development emerged to a much better-defined scenario of molecular and cellular events, so that we can begin to approach atherogenesis as a modifiable rather than an inevitable process. Indeed, changes in diet and exercise, as well as the development of certain drugs that may improve plaque stability (eg, lipid-lowering agents), have made significant inroads in preventing acute atherothrombotic events.6-8 However, to make any progress in developing new therapeutic strategies, a better understanding of the underlying causes of disease progression is required. A large body of evidence indicates that cell death is a major modulating factor of atherogenesis.9-11 The consequences of cell death largely depend on the stage of the plaque and the cell type that is involved. Loss of smooth muscle cells (SMCs), for example, may lead to plaque destabilization and rupture because the tensile strength of the protective cap heavily relies on structural properties determined by the number of SMCs and the collagen they synthesize.11 Macrophages...
actively induce SMC death and destroy collagen fibers by producing matrix metalloproteinases, suggesting that macrophage death is beneficial for plaque stability, but only if cell remnants are efficiently removed.10,12

In a morphological study using electron microscopy, Clarke13 proposed a cell death classification scheme that contains 3 types of death: apoptotic, or type I cell death; autophagic, or type II cell death; and necrotic, or type III cell death. Apoptotic death is characterized by a number of morphological changes such as cell shrinkage, chromatin condensation, nucleosomal DNA degradation, and occurrence of protrusions in the cell membrane (blebbing). Activation of the caspase family of cysteine proteases gives rise to these characteristic features of apoptosis. Protruded blebs are then shed from the cell to form the so-called “apoptotic bodies,” which are degraded in the lysosomes of macrophages or neighboring cells after heterophagocytosis. Numerous studies have identified apoptosis as a prominent feature of advanced, unstable lesions.9,14,15 Apoptosis contrasts with necrosis, which is characterized by cytoplasmic swelling, membrane rupturing, and organelle breakdown, involving remarkably few nuclear changes. Transmission electron microscopic analysis of human carotid plaques showed that many degenerating cells have an ultrastructure typical of necrotic death.16 There is now an increasing awareness that apoptosis and necrosis are not the only cellular mechanisms in the plaque for programmed cell death but that autophagy, a bulk degradation process whereby organelles and cytoplasm are engulfed and targeted to lysosomes for proteolysis, may provide an alternative mechanism determining cell fate in atherosclerosis. To further complicate this matter, recent observations indicate that autophagy and apoptosis are often induced by the same stimuli, share similar effectors and regulators, and are subjected to complex crosstalk mechanisms.17

Definition and Molecular Machinery of Autophagy

**Definition of Autophagy**

Autophagy is an evolutionary conserved process involved in the degradation of long-lived proteins and excess or dysfunctional organelles.18–21 In this process, the cytoplasmic content in the cell is sequestered within double-membrane vacuoles called autophagosomes and subsequently delivered to the lysosome for degradation (Figure 1). Autophagy occurs constitutively at low levels under normal conditions in most cells and is an important house-keeping process. Therefore, autophagy is usually thought of as a survival mechanism. Defective autophagy results in the accumulation of protein aggregates and abnormal organelles, which are toxic to the cell.22–24 The intracellular presence of misfolded or conformationally altered proteins is a main feature of neurodegenerative diseases and may play a key role in their pathogenesis. The occurrence of “basal” autophagy notwithstanding, the autophagic pathway is rapidly activated during starvation when the cell needs to catabolize amino acids and fatty acids from proteins and lipids. Indeed, starvation of mice has been shown to cause substantial upregulation of autophagy in most

tissues. Increasing evidence, however, suggests that autophagy is also upregulated in response to other stresses both extracellular (e.g., hypoxia, treatment with certain hormones or chemicals) and intracellular (e.g., endoplasmic reticulum [ER] stress). Excessive autophagic activity is capable of destroying major proportions of the cytosol and organelles, most noticeably the mitochondria and the ER, finally leading to the total collapse of all cellular functions and induction of autophagic death. This type of death lacks several hallmarks of apoptosis and necrosis, including absence of pronounced chromatin condensation, caspase activation, oligonucleosomal DNA fragmentation, and cellular swelling. Given its role in cell survival, autophagy in dying cells seems to be a sign of failed repair rather than a way for cells to commit suicide by consuming themselves.

Molecular Machinery of Autophagy

Research on autophagy has been ongoing for more than 40 years, but has been restricted by lack of knowledge about the molecular machinery behind this process. Over the last decade, huge advances have been made and genetic screens in yeast have led to the identification of more than 30 autophagy-related genes (Atg genes), many of which have mammalian homologs. In the presence of adequate nutrients, growth factors are able to activate class I phosphatidylinositol-3 kinase (PI3K) proteins, which activate mammalian target of rapamycin (mTOR) via the Akt/PKB pathway. Active mTOR leads to inhibition of the serine/threonine kinase Atg1 (Ulkl in mammalian cells), a key mediator in autophagy induction. If there are inadequate nutrients or in the presence of mTOR inhibitors (e.g., rapamycin), mTOR is not activated, and Atg1/Ulkl is able to form an Atg1 protein kinase autophagy regulatory complex that signals induction of autophagy. Formation of autophagosomes further depends on (1) the assembly of a lipid kinase signaling complex containing class III PI3K that mediates nucleation of the preautophagosomal membrane (phagophore or isolation membrane) and (2) two ubiquitin-like conjugation pathways that stimulate expansion of the isolation membrane. The lipid kinase activity of class III PI3K is thought to create lipid patches of phosphatidylinositol 3-phosphate (PI3-P). These lipid patches recruit proteins from the cytosol for autophagosome biogenesis. Moreover, presence of PI3-P generates significant asymmetries and membrane curvatures in the preautophagosomal membrane. Expansion of the membrane starts by conjugation of Atg12 with the ubiquitin-like protein Atg5 through the action of E1 ubiquitin-activating enzyme Atg7 and E2 ubiquitin-conjugating enzyme Atg10. Finally, Atg16L links with the Atg12-Atg5 conjugate and multimerizes to form a large complex (Figure 1). The second ubiquitin-like protein essential for autophagy is Atg8, better known as microtubule-associated protein light chain 3 (LC3). LC3 is initially synthesized in an unprocessed form, proLC3, but immediately cleaved by Atg4 to produce an active cytosolic form, LC3-I. With the catalysis of Atg7 and a conjugating E2 enzyme, Atg3, LC3-I interacts with phosphatidylethanolamine, an abundant membrane phospholipid, yielding LC3-II (Figure 1). The exact role of LC3-II is not known, but the lipidation reaction leads to a conformational change of LC3 that is critical in autophagosome formation. Once the autophagosome is completed, the Atg12-Atg5-Atg16L complex dissociates from this structure, while Atg4 releases Atg8 on the external lipid bilayer into the cytosol. This uncoating event allows the autophagosome to fuse with lysosomes. The proteins that mediate fusion between the autophagosome and the lysosome are ill defined, although there is an essential role for the small GTP binding protein Rab7 and the lysosome-associated membrane proteins LAMP1 and LAMP2 in this process. Lysis of the autophagosome inner membrane and breakdown of the contents via lysosomal hydrolases occurs in the autolysosome, and the resulting macromolecules are released back into the cytosol through membrane permeases (Figure 1).

Detection of Autophagy in Atherosclerosis

Although several methods have been developed for monitoring autophagy in higher eukaryotes, detection of autophagy in tissue is among the least-developed areas at present. As a consequence, ideal methods for detection of autophagy in atherosclerotic plaques relative to the techniques possible with cells in culture may not exist. It should also be noted that there are no minimal criteria for demonstrating autophagy. Detection guidelines have recently been established, but this standard set of criteria may evolve as new methodologies are developed and present assays of the process are superseded. Overall, we recommend the use of multiple techniques (morphological methods using electron microscopy, combined with immunohistochemistry or molecular techniques) to verify an autophagic response.

Detection of Autophagy via Transmission Electron Microscopy

Because formation of autophagic vacuoles is by far the most important morphological feature of autophagic cells, demonstration of these structures by conventional electron microscopy remains currently the gold standard for assessing autophagy both in tissues and cultured cells. However, interpreting electron microscopy is subjective and it can be difficult to distinguish autophagosomes from lysosomes, endosomes, or other structures in the cell. For example, if mitochondria are swollen or contain precipitates, they can be misinterpreted as autophagosomes. Lipid droplets, as well as electron lucent or empty vacuoles, are also sometimes incorrectly called autophagic vacuoles. Because these vacuoles have no contents, it is not possible to say whether they are autophagic compartments or some other kind of vacuoles. Transmission electron microscopy (TEM) of disintegrating SMCs in the fibrous cap of experimental or human plaques reveals certain features of autophagy such as formation of myelin figures and severe vacuolization (Figure 2). Myelin figures are phospholipids and membrane fragments, often arranged in concentric rings, and represent autophagic degradation of membranous cellular components. These structures are not abundantly present in human plaques but can be found in plaques from cholesterol-fed rabbits or after treatment of SMCs in culture with oxidized lipids (Figure 3).
Detection of Autophagy via Immunohistochemistry or Molecular Techniques

Given the many pitfalls in correctly identifying autophagic vacuoles by TEM, detection of vacuolization in plaque SMCs as a hallmark of autophagy is not sufficient. One of the most useful alternative methods is the analysis of GFP-LC3/Atg8 (eg, in transgenic mice) via fluorescence microscopy, although there are some practical limits. First, a transgenic GFP-LC3 genetic background is needed which excludes the use of human plaques. Direct immunohistochemical detection of LC3 is also possible, but this approach may require overexpression of LC3. Secondly, detection of GFP-LC3 in plaques from transgenic mice can be hampered by strong background fluorescence of extracellular matrix components such as collagen and elastin and does not lend itself well to dynamic assays. In spite of these practical issues, elevated

Figure 2. TEM of dying SMCs in atherosclerotic plaques from cholesterol-fed rabbits (A) or human carotid arteries (B through C). A, Example of a disintegrating SMC in a rabbit plaque adjacent to the endothelial cell (EC) layer. Numerous vacuoles with myelin figures (open arrows) typical of autophagy are detectable. Scale bar = 1 μm. B and C, TEM images of SMCs in the fibrous cap of advanced human plaques illustrating the challenge with which an inexperienced electron microscopist is confronted when trying to identify autophagic compartments. Swollen mitochondria (arrowheads) or other structures in the cell are often incorrectly interpreted as autophagosomes (arrows). By

Figure 3. Transmission electron micrographs of SMCs from rabbit aorta exposed to 25 μmol/L 7-ketocholesterol for 18 hours in low-serum (2%) conditions. A, Untreated control with normal cell morphology. Treatment of SMCs with 7-ketocholesterol causes severe cellular damage and autophagic death, as shown in B through D. 7-Ketocholesterol–induced SMC death is characterized by the formation of numerous autophagosomes that often contain large myelin figures (arrows). Scale bar = 2 μm. N indicates nucleus.

Figure 2 (Continued). Definition, autophagosomes (and autolysosomes) are membrane-bound compartments that contain cytoplasmic material and/or organelles. The presence of a double limiting membrane should not be used as a criterion for the identification of autophagosomes. Sometimes, as shown in A, the limiting membrane of autophagic compartments may not have contrast at all, probably because of lipid extraction during sample preparation. Note the presence of pronounced chromatin condensation in the nucleus (N), which is not a general feature of autophagy. Possibly, the SMCs in B and C are undergoing a mixed type of cell death with characteristics of both apoptosis and autophagy.
levels of LC3-II can be detected in lysates from advanced human plaques via Western blotting (Figure 4). Of note, LC3-II is not detectable in nonatherosclerotic mammary arteries, indicating strong induction of autophagy during atherogenesis. It is, however, worth mentioning that SMCs in culture, even under normal physiological conditions, often show high levels of LC3-II (Figure 4). This finding suggests that autophagy plays an important housekeeping role in SMCs to remove abnormal proteins and other cytoplasmic macromolecules or organelles.

Apart from LC3, detection of granular cytoplasmic ubiquitin inclusions by immunohistochemistry is an attractive non-TEM-based technique, used by many groups, to detect autophagic cell death in heart failure.45–48 Likewise, dying SMCs in the fibrous cap of advanced human plaques show ubiquitinated inclusions in their cytoplasm evocative of autophagic death (Figure 5).41 However, we need to be cautious in the interpretation of experimental evidence supporting autophagic death based on ubiquitin staining. Ubiquitinated aggregates may also result from a malfunction in the autophagic pathway or from structural changes in the protein substrates, halting their degradation.22 In this light, the presence of such inclusions may actually indicate decreased autophagy rather than stimulation of the process.

**Detection of Autophagy in Plaque Macrophages**

Detection of autophagy is even more problematic in plaque macrophages or macrophage-derived foam cells. Macrophages have a strong phagocytic potential, which makes it difficult, if not impossible, to determine via TEM whether the vacuoles in their cytoplasm result from auto- or heterophagocytosis. Moreover, macrophages overexpress lysosomal marker proteins such as cathepsin D or LAMPs (Figure 4) and thus may give rise to false-positive signals after immunohistochemical detection of these enzymes, an approach often used to detect autophagy in other pathologies.49 Previous studies showed that immunohistochemical detection of the autophagy gene beclin 1 can be used as a marker for autophagic death in neurons.50 Beclin 1 is a component of the class III PI3K complex that is required for autophagy.51 Although the protein is highly expressed in macrophages in vitro, it is not differentially expressed in human plaques (Figure 4) and seems to be an unreliable marker for autophagy in many cell types.42 Therefore, owing to (1) the technical and practical limitations for detection, (2) the lack of adequate marker proteins, and (3) the fact that autophagy has been an understudied area in cardiovascular research for many years, the distribution of this process in atherosclerotic plaques has not yet been analyzed in full detail. Considering the physiological and pathological importance of autophagy in atherosclerosis, as described below, the growing interest in this topic will be most rewarding from both basic research and clinical points of view.

**Role of Autophagy in Atherosclerosis**

**Protective Effects of Autophagy in Atherosclerosis**

The role of autophagy in atherosclerosis is poorly understood. Because autophagy is well recognized as a survival mechanism and not as a death pathway,50 it is tempting to speculate that autophagy of SMCs in the fibrous cap of advanced lesions is in the first place an important mechanism underlying plaque stability. Autophagy most likely safeguards plaque cells against oxidative stress, a hallmark of advanced atherosclerotic lesions, by degrading the damaged material, in
particular polarized mitochondria in the very early stages before cytochrome c release occurs. In this way, successful autophagy of the damaged components contributes to cellular recovery. If autophagy is not sufficient for the removal of the cellular damage (e.g., in the case of severe oxidative stress), leakage of intramitochondrial components such as cytochrome c may induce apoptosis through activation of the caspase cascade. Moreover, oxidative damage of the lysosomal membrane often results in cytosolic leakage of potent hydrolases, which could cause substantial cytosolic damage, even at the neutral cytosolic pH, followed by apoptosis. Severe oxidative stress combined with autophagy may also lead to formation of ceroid, a complex of protein associated with oxidized lipids. Ceroid deposits cannot be degraded by lysosomal hydrolases and might lead to preferential allocation of lysosomal enzymes to ceroid-loaded lysosomes at the expense of active autolysosomes, which, in turn, would lead to progressive inhibition of autophagy and the induction of apoptosis. Besides its important antiapoptotic role in atherosclerotic plaques, autophagy may also downregulate apoB-containing lipoproteins in the circulation. Dietary polyunsaturated fatty acids induce the appearance of intracellular aggregates of apoB in the liver. These aggregates slowly degrade by an autophagic process, thereby inhibiting export of apoB lipoproteins by hepatocytes and infiltration of these compounds in the vessel wall.

Another example that illustrates the protective effects of autophagy in atherosclerosis comes from a recent pharmacological study using statins and 7-ketocholesterol. Statins protect patients from myocardial infarction, although they are known to induce SMC apoptosis in a dose-dependent manner. SMC death induced by low concentrations of statins is not stimulated, but attenuated by the autophagy inducer 7-ketocholesterol. Indeed, fluvastatin fails to raise caspase activity in SMCs treated with 7-ketocholesterol, suggesting that the activation of autophagy interferes with the statin-induced apoptotic pathway. The mechanisms of this suppression are not yet understood, but it has been proposed that the engulfment of defective mitochondria by autophagosomes
limits the release of proapoptotic proteins such as cytochrome c and apoptosis inducing factor into the cytosol.\textsuperscript{54}

Recently, Pan et al\textsuperscript{55} reported a role for autophagy in late-stage quality control of apolipoprotein (apo)B, a glycoprotein secreted by the liver and an essential component of lipoproteins in human plasma. Retention, or trapping, of apoB-containing lipoproteins within the arterial wall is the key initiating event in the pathogenesis of atherosclerosis.\textsuperscript{2} Three processes have been described that regulate posttranslational degradation of apoB: ER-associated degradation (ERAD), post-ER presecretory proteolysis (PERPP), and reuptake via interactions of nascent apoB-particles with low-density lipoprotein (LDL) receptors and heparin sulfate proteoglycans. PERPP is stimulated under a number of common metabolic conditions, such as the uptake of polyunsaturated fatty acids (PUFAs) by hepatocytes\textsuperscript{56} and high levels of insulin,\textsuperscript{57} which occur after eating. The addition of PUFAs to cultured hepatocytes enhances the destruction of apoB via PERPP through the elaboration of lipid peroxides that damage and aggregate apoB.\textsuperscript{55,58} Aggregated apoB then becomes directed into lysosomes via autophagosomes, without ever leaving the cell (Figure 6). These findings may explain the well-known observation that diets rich in PUFAs, especially those of the omega-3 class found in marine oils, lower plasma levels of very-low-density lipoprotein and especially those of the omega-3 class found in marine oils, lower plasma levels of very-low-density lipoprotein and LDL, the major apoB lipoproteins secreted by the liver, and inhibit progression of atherosclerosis.\textsuperscript{59}

### Detrimental Effects of Autophagy in Atherosclerosis

Apart from its protective role, autophagy in atherosclerosis is responsible for the formation of ceroid, an insoluble complex of protein associated with oxidized lipids found in all human atherosclerotic lesions.\textsuperscript{60} Hydrogen peroxide (H$_2$O$_2$) generated by mitochondria and other organelles permeates in the lumen of secondary lysosomes. These lysosomes contain iron derived from cellular structures undergoing autophagic degradation.\textsuperscript{61} The interaction between reactive ferrous iron and H$_2$O$_2$ results, via Fenton reactions, in the generation of hydroxyl radicals inducing lipid peroxidation and eventually intermolecular crosslinking and ceroid formation.\textsuperscript{62} Indeed, Lee et al\textsuperscript{63} reported that iron and ceroid deposits colocalize either extracellularly or intracellularly in foam cell-like macrophages or SMCs of advanced plaques. Many cells in advanced human plaques contain a large number of ceroid-containing lysosomes to which more and more lysosomal enzymes are directed in a useless effort to degrade ceroid. These lysosomal enzymes are lost for useful purposes (eg, for the degradation of newly autophagocytosed material),\textsuperscript{64} resulting in impaired autophagy and the induction of apoptosis (Figure 6). Impaired autophagy stimulates further accumulation of damaged mitochondria, increased ROS generation, and enhanced ceroid formation.\textsuperscript{61} Interestingly, continuous autophagic intrlysosomal degradation of ferruginous materials combined with the formation of H$_2$O$_2$ and the peroxidation of the lysosomal membrane might result in its subsequent rupture, especially under conditions of severe oxidative stress, with release of harmful lysosomal enzymes.\textsuperscript{61,64} If of limited magnitude, such release can induce "reparative autophagy,"\textsuperscript{65} causing additional accumulation of iron and non-degradable oxidation products such as ceroid. Finally, these events sensitize cells to undergo apoptosis as released lysosomal enzymes can attack other proteins and mitochondria, triggering cytochrome c release with an amplification of the apoptotic program (Figure 6).\textsuperscript{61}

In contrast to basal autophagy, excessively stimulated autophagy may cause autophagic SMC death,\textsuperscript{60,63} which in turn results in plaque destabilization owing to the reduced synthesis of collagen and thinning of the fibrous cap. Also, autophagic death of endothelial cells may be detrimental for the structure of the plaque as endothelial injury and/or death represents a primary mechanism for acute clinical events by promoting lesional thrombosis.

### Potential Factors That Stimulate Autophagy in Atherosclerosis

Although much progress in identifying autophagy in atherosclerosis has yet to be made, in vitro observations suggest that various atherosclerosis-related factors stimulate autophagy in plaque cells.

#### Stimulation of Autophagy by Oxidized Lipids

During atherogenesis, excess amounts of LDLs infiltrate atherosclerosis-prone regions of the artery, where they are modified through oxidation or enzymatic attack.\textsuperscript{2} Oxidative degradation of infiltrated lipids generates a variety of bioactive intermediates and end-products.\textsuperscript{66} These include lipid hydroperoxides and lipid peroxidation-derived aldehydes such as malondialdehyde, 4-hydroxyxenal (4-HNE), or 1-palmitoyl-2-oxovaleryl phosphatidylcholine (POVPC). Exposure of SMCs to 4-HNE leads to the modification of several proteins, as detected by anti–protein–4-HNE antibodies or protein-bound radioactivity in [H]$^3$H]-treated cells, and stimulates processing of LC3 into the autophagosome-specific isoform LC3-II.\textsuperscript{67} Modification of proteins by 4-HNE may be harmful not only because it disrupts the function of the protein but also because it leads to the accumulation of inactive or crosslinked proteins, which must be removed to prevent further toxicity. Protein–4-HNE removal is not inhibited with the proteasome inhibitor lactacystin, suggesting that proteasome-mediated degradation may be a minor pathway for the removal of proteins modified by 4-HNE.\textsuperscript{67} This finding is consistent with previous work showing that 4-HNE inhibits the proteasome and that the proteasome is unable to degrade proteins heavily modified by 4-HNE.\textsuperscript{68} In contrast, inhibition of the lysosomal–autophagy pathway by 3-methyladenine leads to significantly greater accumulation of protein–4-HNE adducts, indicating that protein–aldehyde adducts may be degraded as part of the autophagic response.\textsuperscript{65} Moreover, removal of protein–4-HNE adducts is accelerated by the autophagy inhibitor rapamycin and decreased by the autophagy inhibitor insulin.\textsuperscript{67} At the ultrastructural level, 4-HNE–treated cells display extensive vacuole formation, pinocytic body formation, crescent-shaped phagophores, and multilamellar vesicles. The mechanisms by which 4-HNE or protein–4-HNE adducts trigger autophagy remain unclear. It is likely that autophagy is induced by increasing ROS generation and oxidative injury.
Indeed, oxidative stress is a well-known stimulus of autophagy to facilitate the removal of damaged organelles.\(^{52}\) However, products of lipid peroxidation could also directly trigger autophagic signaling through formation of electrophile-modified or crosslinked proteins.

In analogy with 4-HNE, autophagy can be triggered by oxidized (ox)LDL. Exposure of EA.hy926 endothelial cells in vitro to oxLDL intensifies autophagy as compared to cultures treated with native LDL or medium alone.\(^{69}\) Moreover, 7-ketocholesterol, one of the major oxysterols present in oxLDL, not only triggers oxidative damage and protein–4-HNE modification but also extensive vacuolization (Figure 3), intense protein ubiquitination, and LC3-II formation in SMCs.\(^{41}\) It is also important to note that lipid-laden SMCs in human plaques or cultured SMCs treated with aggregated LDL upregulate death-associated protein (DAP) kinase,\(^{70}\) a positive mediator of apoptotic cell death that controls membrane blebbing and the formation of autophagic vesicles.\(^{71}\) Whether DAP kinase in plaque SMCs stimulates apoptosis, autophagy, or both is unclear. It has been reported that DAP kinase acts as a survival factor in SMCs and that acute depletion of this cytoprotective kinase induces apoptosis in cultured cells, even when grown in normal conditions.\(^{72}\)

### Stimulation of Autophagy by ER Stress

ER stress and activation of the unfolded protein response (UPR) are markedly increased in macrophages from both early and advanced atherosclerotic lesions.\(^{73}\) Among the potential initiators of UPR, it is worthwhile to mention the accumulation of intracellular free cholesterol, a strong inducer of macrophage apoptosis.\(^{74}\) Accumulation of free cholesterol can be observed in early-lesion-resident macrophage foam cells, but these cells do not undergo apoptotic cell death. Indeed, ER stress has recently been shown to induce autophagy to protect against cell death.\(^{29}\) A major UPR-upregulated target protein is the 78-kDa glucose-regulated protein, GRP78, an ER molecular chaperone also referred to as BiP. Evidence is now emerging that GRP78/BiP is an obligatory component of autophagy in mammalian cells because knockdown of this protein leads to ER expansion and inhibition of autophagosome formation.\(^{75}\)

### Stimulation of Autophagy by Inflammation

T lymphocytes infiltrate the arterial intima at an early stage in plaque formation together with monocytes and release proinflammatory cytokines such as interferon (IFN)-γ, interleukin (IL)-2, and tumor necrosis factor (TNF)-α.\(^{76}\) Recent reports suggest that cytokines take part in modulation of autophagy.\(^{77,78}\) Indeed, TNF-α stimulates expression of LC3 and the autophagy gene beclin 1 in plaque SMCs through Akt/PKB and c-jun N-terminal signal pathways, which results in autophagic SMC death.\(^{79}\) Furthermore, IFN-γ induces autophagy in macrophages\(^{80}\) and in nonimmune cells\(^{71}\) through the immunity-related GTPase Irgm1, also known as LRG-47.\(^{77}\) Other immune modulators such as the Th2 cytokines IL-4 and IL-13 have the potential to act as suppressors of autophagy because they stimulate type I PI3K and thus also mTOR.\(^{77}\) Given the predominantly proinflammatory Th1 type immune response in atherosclerosis,\(^{76}\) inflammatory cells in advanced plaques serve as an important source of proautophagic stimuli.

Besides cytokine production, inducible nitric oxide synthase (iNOS) is overexpressed in macrophage-derived foam cells so that cytotoxic amounts of NO are produced.\(^{81}\) NO may contribute to oxidative stress and tissue damage through formation of peroxynitrite. Once formed, peroxynitrite can potentially oxidize and/or damage a variety of biomolecules, including polyunsaturated fatty acids, sulfhydryl groups, and cellular DNA, which may evoke an autophagic response to remove the damaged material.\(^{52}\)

### Stimulation of Autophagy by Hypoxia or Metabolic Stress

Hypoxia and hypoxia-induced cell death are common features of advanced human atherosclerotic plaques caused by inadequate vascularization that results in nutrient, growth factor, and oxygen deprivation.\(^{82}\) The precise mechanisms of hypoxia-induced cell death remain unclear because apoptosis, necrosis, and autophagy have all been reported in response to hypoxic stress.\(^{26,83}\) Although nutrient starvation is a powerful inducer of autophagy,\(^{25}\) metabolic stress (hypoxia combined with nutrient deprivation) induces organelle, protein, and DNA damage that potently stimulate apoptosis.\(^{84}\) Recent evidence, however, suggests that autophagy mitigates metabolic stress to protect the cell against severe cellular damage.\(^{85}\) Autophagy not only is required as an alternate means to generate ATP during periods of starvation but also has a role in maintaining homeostasis through protein and organelle quality control. These functions of autophagy may be particularly critical in situations of metabolic stress, where ATP is limiting and cellular damage accumulates at an accelerated rate. Because SMCs in the fibrous cap of advanced human plaques are surrounded by a thick layer of basal lamina,\(^{86}\) it is conceivable that autophagy in these “caged” cells is stimulated as a result of starvation effects. It should be noted, however, that the ability of autophagy to limit apoptosis is modest, if apparent at all, in the context of an intact apoptotic response.\(^{84}\)

### Selective Depletion of Macrophages in Atherosclerotic Plaques via Autophagy Induction

Because macrophages play a central role in atherosclerotic plaque destabilization, selective induction of macrophage death now gains increasing attention in cardiovascular medicine to stabilize vulnerable, rupture-prone lesions.\(^{87}\) Compared with apoptosis or necrosis, autophagy seems to be an interesting type of death to eliminate macrophages in atherosclerotic plaques, at least from a theoretical point of view, because autophagic cells literally digest themselves to death. As a consequence, the cytoplasmic content progressively decreases so that activation of inflammatory responses, the release of matrix-degrading proteases, and the deposition of necrotic debris after postautophagic necrosis are minimal.

### Selective Induction of Autophagic Death in Plaque Macrophages via mTOR-Dependent Pathways

A large number of drugs, signaling complexes, and pathways are able to regulate the initiation and maturation of autoph-
In line with these findings, stent-based Atg13 association is required for the formation of autophagy in plaques from cholesterol-fed rabbits. 

**Figure 7.** Schematic overview of different signaling pathways involved in modulation of autophagy.

One such pathway involves mTOR, a serine/threonine kinase and an evolutionarily conserved member of the phosphoinositide kinase–related kinase family that controls the response to changes in nutrients such as amino acids (mainly leucine), growth factors, insulin, and mitogens. Blocking mTOR function using rapamycin or its analogs (rapalogs) mimics the deprivation of amino acids and growth factors and has a cytostatic effect on proliferating cells both in vitro and in vivo. As a consequence, rapamycin has been used successfully as an immunosuppressant for organ transplantation (by blocking proliferation of activated T cells) and as a therapeutic agent in the prevention of restenosis after balloon angioplasty and stenting (by blocking proliferation and migration of SMCs). Recent results from clinical trials suggest that rapamycin (or rapalogs) may also be useful for the treatment of certain types of cancer. In addition to its well-known effects on cell growth, inhibition of mTOR may lead to autophagic cell death through activation and/or upregulation of some Atg proteins (Figure 7, a). Atg13, for example, is rapidly dephosphorylated on inhibition of the mTOR pathway, stimulating its affinity for Atg1. This Atg1-Atg13 association is required for the formation of autophagosomes (Figure 1). In line with these findings, stent-based delivery of the rapamycin derivative everolimus in atherosclerotic plaques from cholesterol-fed rabbits leads to a marked reduction in macrophage content via autophagic cell death without altering the amount of SMCs. In vitro studies showed that everolimus induces inhibition of de novo protein synthesis in both macrophages and SMCs by dephosphorylating the downstream mTOR target p70 S6 kinase, followed by bulk degradation of long-lived proteins, processing of LC3, and cytoplasmic vacuolization in macrophages but not in SMCs. Interestingly, apart from the mTOR pathway, local administration of the protein synthesis inhibitor cycloheximide induces selective macrophage death in plaques from cholesterol-fed rabbits, but in contrast to everolimus, apoptosis and not autophagy is induced. Measurements of oxygen consumption, as well as immunodetection of markers for DNA synthesis/repair, indicate that plaque macrophages are metabolically highly active and thus more sensitive to protein synthesis inhibitors as compared to SMCs. Moreover, inhibition of translation in SMCs by rapamycin induces a modulation toward a differentiated, quiescent, contractile phenotype through upregulation of smooth muscle α-actin, calponin, and myosin heavy chain, which may render SMCs relatively insensitive to cell death mediated by inhibition of protein translation. Therefore, inhibition of translation rather than differential expression of cell death proteins seems to be a major trigger that drives selective induction of macrophage death. However, dephosphorylation of the downstream mTOR target p70 S6 kinase after everolimus treatment, most notably dephosphorylation at Thr389, which is the principal site of rapamycin-induced p70 S6 kinase inactivation, occurs at very low concentrations (0.1 to 1 nmol/L), whereas induction of macrophage death only occurs after treatment with relatively high everolimus concentrations (>3 μmol/L). Accordingly, one may assume that inhibition of other intracellular proteins, but not inhibition of mTOR (aspecific inhibition), is responsible for everolimus-induced macrophage death. Because the viability of macrophages is not affected using the same concentration of tacrolimus, an mTOR-independent everolimus analog, this assumption is unlikely. Moreover, mTOR gene silencing after transfection of mTOR-specific small interfering RNA is associated with selective induction of macrophage cell death. It is likely that other mTOR-mediated pathways, but not dephosphorylation of p70 S6 kinase, are responsible for selective induction of macrophage death.

**Selective Induction of Autophagic Death in Plaque Macrophages via mTOR-Independent Pathways**

Besides mTOR inhibitors, several compounds might serve as ideal drug candidates. For example, the imidazoquinoline compound imiquimod stimulates autophagy after binding to Toll-like receptor (TLR)7 (Figure 7, b). This protein is expressed only in immune cells so that imiquimod is able to induce autophagic death in macrophages, but not in SMCs (I.D.M., unpublished data, 2008). However, a simple correlation between the downstream signaling pathways induced by TLR7 and autophagy cannot be drawn. TLR7 signaling can lead to mitogen-activated protein kinase (c-Jun N-terminal kinase [JNK], p38, and extracellular signal-regulated kinase [Erk]). JNK in turn might activate autophagy via modulation of Bcl-2–Beclin 1 interactions and Beclin 1 activity, although p38 and Erk have been implicated in autophagy both as a positive and a negative factor. Alternative possibilities for TLR7 activation of autophagy are induction of type I IFN (IFN-α and -β) and nuclear factor κB activation. A pivotal role for these mechanisms is controversial as IFN-α/β does not induce autophagy in RAW264.7 macrophages, whereas activation of nuclear factor κB more likely counteracts and represses autophagy.

Inhibition of the 1,4,5-inositol triphosphate (IP₃) receptor (IP₃R) with a specific antagonist, xestospongin B, is a strong stimulus of autophagy (Figure 7, c). Xestospongin B induces autophagy through a pathway that requires the...
obligate contribution of the autophagy proteins beclin-1, Atg5, Atg10, Atg12, and hVps34 yet is inhibited by Bcl-2 or Bcl-X_{L}, 2 proteins that physically interact with IP_{3}R. In addition to xestospongin B, autophagy can be induced by lithium, an inhibitor of inositol monophosphatase, that after administration leads to depletion of free inositol and reduced levels of IP_{3} (Figure 7, d). Recent experiments in our laboratory showed that treatment of atherosclerotic plaques with lithium triggers selective macrophage death (I.D.M., unpublished data, 2008).

Recently, an mTOR-independent autophagy pathway involving cAMP/Ca^{2+}/calpains/G_{50a} has been described. Pharmacological inhibition of this pathway induces autophagy. This can be achieved by e.g., the L-type Ca^{2+} channel blocker verapamil. Whether this pathway affects macrophages or other cell types in the plaque is presently unknown.

Selective Depletion of Macrophages in Atherosclerotic Plaques via Modulation of Sterol Levels: Role of Autophagy

One of the best known approaches to clear macrophages in atherosclerotic plaques is via dietary lipid lowering. Until recently, it was unclear whether the loss of macrophages resulted from decreased accumulation/replication, increased cell death, or a combination of both. Recent evidence suggests that loss of macrophages in plaques from cholesterol-fed rabbits after dietary lipid lowering is not related to induction of macrophage apoptosis but mainly a consequence of impaired monocyte recruitment followed by decreased macrophage replication. However, these mechanisms may not fully explain the loss of resident macrophages. We therefore believe that additional mechanisms cannot be excluded. Indeed, based on in vivo studies, Llodra et al. reported that monocyte-derived cells can emigrate from the plaque during lesion regression. Furthermore, depletion of cholesterol can induce autophagy. The mechanism by which this happens is unclear, although disruption of cholesterol-rich lipid rafts might be involved (Figure 7, e). Among several proteins engaged in autophagic regulation, basal and several proteins engaged in autophagic regulation, basal and growth factor–induced Akt activity is known to be dependent on lipid raft integrity. Thus, a potential scenario would be that cholesterol depletion disrupts membrane rafts, and the resultant downregulation of Akt activity leads to suppression of mTOR and autophagy induction. Because only drastic cholesterol depletion methods induce autophagy of cells in vitro, and LDL cholesterol levels of cholesterol-fed rabbits do not change during the first weeks of dietary lipid lowering, we do not believe that induction of autophagy is involved in macrophage clearance, at least not early after cholesterol withdrawal.

Phytosterols or plant sterols decrease plasma cholesterol levels by competing with cholesterol for incorporation into micelles, thereby decreasing the intestinal absorption of cholesterol. For this reason, they are used as food supplement, for example in certain margarines. In contrast to cholesterol, the level of phytosterols in plasma and atherosclerotic plaques of most humans is very low, but in patients with sitosterolemia, plasma levels of plant sterols reach very high levels, leading to premature and severe coronary artery atherosclerotic disease. Phytosterols are very poor substrates for sterol O-acyltransferase 1 (SOAT1), a sterol-esterifying enzyme that prevents cell death by inhibiting intercalation of free sterols into cell membranes. Accordingly, free phytosterols may accumulate in the plaque, particularly in macrophages after phagocytosis, thereby inducing a selective caspase-independent type of macrophage death in which necrosis and autophagy are involved (Figure 7, f).

Induction of Autophagic Death in Plaque Macrophages: A Problem of Specificity

The development of strategies that mediate depletion of macrophages from atherosclerotic plaques via autophagy is often hampered by lack of specificity or unexpected adverse effects. The pancaspase inhibitor z-VAD-fmk, for example, induces autophagy and necrotic cell death in J774A.1 and RAW264.7 macrophages, as well as in IFN-γ–primed primary mouse peritoneal macrophages, but not in vascular SMCs or C2C12 myoblasts. Autophagy actually serves as a cell survival mechanism to protect against z-VAD-fmk–induced necrotic cell death. However, z-VAD-fmk–treated J774A.1 macrophages overexpress and secrete several chemokines and cytokines, including TNF-α. The combination of z-VAD-fmk and TNF-α, but not TNF-α alone, induces SMC necrosis. In this regard, z-VAD-fmk is detrimental and not beneficial for atherosclerotic plaque stability because of stimulation of inflammatory responses and indirect induction of SMC death. Another compound with proautophagic potential is concanavalin A (Con A), a plant lectin isolated from Jack bean seeds that, once bound to the mannose moiety of cell membrane glycoproteins, is internalized preferentially to the mitochondria. This may cause changes in the mitochondrial membrane permeability that stimulates a BNIP3-dependent autophagic pathway (Figure 7, g). Although monocytes/lymphocytes are highly sensitive to Con A because of their high content of mannose-containing moiety on the cell membrane, Con A does not induce selective macrophage death (I.D.M., unpublished data, 2008).

Conclusion and Future Perspectives

A growing body of in vitro evidence suggests that autophagy occurs in atherosclerotic plaques. However, despite the astonishing number of recent connections to various pathophysiological situations such as neurodegeneration, cancer, and cardiac myopathies, the in vivo distribution of autophagy in atherosclerosis has yet to be teased out. Good marker proteins are needed first to determine unambiguously in which cell types of the plaque autophagy can be found and whether induction of autophagy is associated with early or late lesions. Furthermore, it is unclear whether autophagy is harmful or protective in atherosclerotic plaques. Most likely, autophagy under basal conditions plays an important role in cellular housekeeping, whereas induced autophagy may function as a death pathway. Nonetheless, several questions remain unanswered. For example, how long can we activate autophagy without detrimental consequences for the cell? Does autophagy have a significant impact on other cell death
pathways in the plaque, and are senescent SMCs less capable of inducing autophagy as compared to other cell types? Crossbreeding of mouse models for atherosclerosis (eg, ApoE or LDL receptor knockout animals) with autophagy-deficient mice (eg, conditional Atg5 knockout animals) will undoubtedly help to answer these questions and may shed more light on the potential role of autophagy in atherosclerosis. The challenge for clinicians will be to turn on the protective effects of autophagy without activating unwanted death pathways. So far, pharmacological induction of autophagic cell death in macrophages is believed to be the preferred type of death to deplete this type of cells from atherosclerotic plaques.12 However, it should be noted that this approach might involve certain adverse effects. In vitro, autophagic macrophages produce proinflammatory cytokines such as TNF-α and IL-6,129 suggesting that the autophagic process is not immunologically silent. Moreover, it remains unclear what happens with the large amount of oxidized lipids in the cytoplasm of macrophage-derived foam cells undergoing autophagy. It is tempting to speculate that lipids in the cytosol are not adequately digested during autophagy owing to overload or exhaustion of lysosomal enzymes. Furthermore, an increase rather than a decrease in foam cell formation can be expected in macrophages undergoing autophagy because protein degradation, as well as the decline of protein synthesis, in autophagic cells readily blocks the utilization of lipids for lipid–protein conjugation, which in turn results in the formation of lipid droplets.87 These lipid droplets can be spilled out in the microenvironment of the plaque when the autophagic cell collapses, thereby attracting new mononuclear cells from the circulation. Finally, it is of note that some experimental drugs used in anticancer or other therapies can translocate into the circulation, where they might facilitate autophagic death of endothelial cells and atherothrombotic events. Endostatin, for example, a potent inhibitor of neovascularization and tumor growth, triggers autophagic cell death in human EA.hy926 endothelial cells.129 Also, water-soluble nanomaterials such as fullerenes, which currently under development for targeted drug delivery into the brain or cancer tissue, markedly increase the accumulation of polyubiquitinated proteins and induce autophagic cell death in human umbilical vein endothelial cells.130 Overall, these preliminary data indicate that further research is needed before drugs with proautophagic potential can be used in the clinic to treat atherosclerosis and other human diseases.

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Disclosures
None.

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